Advances in Brief

Taxol Blocks Processes Essential for Prostate Tumor Cell (PC-3 ML) Invasion and Metastases¹

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Abstract

We have examined the antimetastatic effects of taxol on a PC-3 human prostatic tumor variant (PC-3 ML) which metastasizes to the lumbar vertebrae in severe combined immunodeficiency-carrying (SCID) mice. Immunofluorescence labeling indicated that taxol (0.5 to 1.0 μM for 6 h) produced an abnormal bundling of microtubules in a dosage-dependent manner. Slot blotting and gelatinase assays revealed that taxol inhibited secretion of the M₇, 72,000 and M₉, 92,000 type IV collagenases plus a M₇, 57,000 gelatinase. Radiolabeling measurements confirmed that the drug inhibited both the secretion and the synthesis of the M₇, 72,000 collagenase. Taxol also blocked total protein secretion but did not influence total protein synthesis or turnover. Boyden chamber chemotactic assays further showed that taxol (0.5 to 1.0 μM) inhibited invasion of Matrigel. More importantly, studies in SCID mice demonstrated that taxol (50 to 250 mg/ml/day) blocked the establishment, growth, and long-term survival of PC-3 ML cells.

Introduction

Taxol, an alkaloid from Japanese and Pacific yew, is a potent drug with tremendous potential in the therapeutic treatment of cancer. Horwitz et al. (1) and others (2) reported that the principle effect of taxol was to stabilize microtubule structures. One striking effect of taxol was that it induced an abnormal bundling of microtubules in cells, blocking cell division (2). Basically, taxol binds tubulin to form abnormally stable taxol microtubules (3-5).

In the past few years, taxol has been successfully tested on patients with cancer (6-12), specifically in the treatment of malignant melanoma (7) and ovarian carcinoma (8, 9). The ovarian cancer studies indicated that taxol was active at dosages of 110 to 175 mg/m² (8). The drug had significant activity against melanoma with some non-life-threatening side effects (i.e., nausea, vomiting) (7). Holmes et al. (2) have recently shown that the drug can be used to treat metastatic breast cancer in phase I and II trials. Taxol, therefore, may be of value in the treatment of a wide range of cancers, including colon and prostate cancer. Future trials need to assess drug treatment protocols, optimum dosages, and dose-limiting toxic effects. In addition, the utility of taxol in treating multidrug-resistant, hormone-resistant and metastatic tumor lesions should be addressed.

In attempts to study prostate cancer metastases, we have developed a SCID² mouse model for investigating metastases to the bone. A variant (PC-3 ML) of human prostate PC-3 tumor cells has been isolated which preferentially metastasizes to the lumbar vertebrae with ≥80% efficiency following i.v. injection via the tail vein in SCID mice (13). In this paper, we have utilized the SCID mouse model to test whether taxol might have therapeutic utility in the prevention or treatment of metastatic bone cancer.

We describe novel observations on the antimetumorigenic effects of taxol on biological processes (i.e., protease secretion) important for tumor cell invasion in vitro. More importantly, we show that taxol is effective with marginal toxicity in the treatment of multidrug-resistant, metastatic bone tumors formed by the PC-3 ML variant in SCIDs.

Materials and Methods

The isolation and culturing of human PC-3 cells and DU 145 cells have been detailed in previous studies (13, 14). The cells were plated on Matrigel (2 mg/ml) unless indicated differently. We have described the methods for preparation and use of PC-3 ML CM (15), the Boyden chamber chemotactic assays, and the preparation and use of Matrigel (14, 15). The slot blotting and immunofluorescence techniques for labeling cells also have been developed previously (14, 15). As described in the table legends, we labeled the cells with [3H]methionine using standard procedures (13).

For the mouse studies, the cells were injected i.v. and s.c. in SCID mice using published methods (13). The resulting tumor tissue was examined by light microscopy following histological preparation of tissue (13). Cryosections were prepared for antibody labeling and immunofluorescence studies according to the methods described by Brandtzaeg (16). The monoclonal β-tubulin antibodies and polyclonal M₇, 72,000 type IV collagenase antibodies used in these studies have been characterized previously (14, 15). Western blots and slot blots with the type IV collagenase antibodies used at a dilution of 1:200 (=1 μg/ml) showed that the antibodies detected as little as 0.10 μg/ml protease and showed a linear increase in labeling up to about 10 μg/ml of purified protease. The levels measured in this paper were within this linear range.

Gelatinase zymography assays were carried out according to the procedures of Heussen and Dowdle (17). All protein measurements were with a Bio-Rad kit using their procedures. All the reagents were from Sigma Chemical Co. unless stated otherwise.

Taxol (Sigma Chemical Co., St. Louis, MO) was solubilized in castor oil and aliquots (i.e., 5-10 μl) were added to solutions. Similar amounts of castor oil were added in control experiments. In general, care was taken to ensure animal welfare on a daily basis.

Toxicity end points were determined by exposing mice to taxol at increased dosages (25, 50, 100, 200, 250, 300, 350 mg/m²/day) for 5 days. Three mice were tested per dosage. Lethargy, sickness, and hyperventilation were observed at dosages greater than 250 mg/m²/day and the animals sacrificed by cervical dislocation. No histopathological criterion was used to assess toxicity.

Results and Discussion

Micromolar levels of taxol had a dramatic effect on the microtubule networks of PC-3 ML cells cultured on Matrigel.
Following immunofluorescence labeling with β-tubulin antibodies, Fig. 1 compares the normal microtubule arrays emanating from a central microtubule-organizing center in untreated cells (Fig. 1A); and the microtubule bundles observed in the taxol-treated cells (Fig. 1B), where the cells were exposed to 0.5 μM taxol for 6 h at 37°C. Note the discontinuous nature of the microtubules located in the peripheral margins of the taxol-treated cells.

The dose-dependent effects of taxol on microtubule bundling were summarized in Table 1. The amount of bundling observed by immunofluorescence labeling was minimal or nonexistent at low levels of taxol tested (<0.05 μM for 1 to 6 h). In contrast, at higher dosages (>0.5 μM for periods of 6 h) 100% of the cells exhibited microtubule bundling. A similar response to taxol was observed in the other PC-3 variants (i.e., 3 × noninvasive) and DU 145 cells (data not shown). Moreover, these results agree closely with that originally reported for other cells (1, 2).

Therefore, we believe that the limiting dosage-dependent parameter in these studies was the time for diffusion of taxol across the cell membranes. Although radio labeled drug uptake studies were not done, drug uptake in the cells appeared to be relatively slow and largely irreversible. For example, microtubule bundling was observed following exposure to 0.5 μM taxol for 6 h and removal of the drug from the medium for 48 h.

Effects of Taxol on Protease Secretion. Previously, we and others (13, 15, 17, 18) have reported that type IV collagenase was secreted by human prostate tumor cells. Plating these cells on type IV collagen (14), in the presence of their own CM (13), significantly elevated the levels of type IV collagenase secreted. In this paper, the cells were plated on Matrigel (2 mg/ml) which stimulated the cells to secrete collagenase (i.e., it had a similar effect as type IV collagen). The inhibitory influence of taxol on type IV collagenase secretion was then examined by slot blotting of the culture medium (Fig. 2). Slot blots with antibodies raised against a M, 72,000 type IV collagenase indicated that the protease was normally secreted by PC-3 ML cells in response to the PC-3 ML CM (10 mg/ml for 6 h). If the cells were pretreated with taxol (0.5 and 1.0 μM for 6 h) prior to stimulation of secretion with conditioned medium, the drug blocked the secretion of the M, 72,000 type IV collagenase. Slot blots of whole cell extracts from the same cells confirmed that type IV collagenase was in fact present in the taxol-treated PC-3 ML cells (Fig. 2). A standard curve assay with affinity-purified collagenase showed a linear antibody (1:200 dilution) detection range from 0.01 to 30 μg, with saturation at 30 μg.

Control experiments showed that when 1 μM taxol was added to PC-3 ML CM (10 mg/ml) prior to (for 3 h) and during the experiment, the drug did not block the ability of CMs to stimulate protease release (data not shown). We interpret this to mean that taxol probably does not bind or interfere with processes activated by factors in the CM per se (14).

Gelatinase assays confirmed that taxol (0.5 μM for 6 h) inhibited the secretion of two high molecular weight gelatinases, including a faint band at M, 92,000 and a pronounced band at M, 72,000 (Fig. 3). Secretion of lower molecular weight gelati-
Table 2  Pulse labeling experiments measuring effects of taxol on synthesis (cpm x 1000 ± SD)

<table>
<thead>
<tr>
<th>Protein synthesis</th>
<th>Collagenase synthesis</th>
<th>Collagenase turnover</th>
<th>Collagenase secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>350 ± 5.1</td>
<td>330 ± 4.0</td>
<td>295 ± 1.5</td>
</tr>
<tr>
<td>3 h</td>
<td>350 ± 5.1</td>
<td>330 ± 4.0</td>
<td>295 ± 1.5</td>
</tr>
<tr>
<td>6 h</td>
<td>350 ± 5.1</td>
<td>330 ± 4.0</td>
<td>295 ± 1.5</td>
</tr>
</tbody>
</table>

* SFM, serum-free medium.

The cells were pulse labeled as in the above experiments and exposed to SFM containing 1.0 µM taxol plus 10 mg/ml PC-3 ML CM for 0, 3, and 6 h. 3H-collagenase was immunoprecipitated from whole cell extracts and the medium of the same cells.

Table 3  Effects of taxol on total protein and collagenase secretion (cpm x 1000 ± SD)

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Total protein secreted</th>
<th>Total collagenase secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>360 ± 0.20</td>
<td>40 ± 0.40</td>
</tr>
<tr>
<td>6</td>
<td>305 ± 0.20</td>
<td>30 ± 0.15</td>
</tr>
<tr>
<td>9</td>
<td>0.2 ± 0.09</td>
<td>0.06 ± 0.02</td>
</tr>
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</table>

Table 4  Effects of taxol on PC-3 ML cell invasion

<table>
<thead>
<tr>
<th>Taxol (µM)</th>
<th>1 h</th>
<th>3 h</th>
<th>6 h</th>
<th>9 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.10</td>
<td>0.10</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>0.05</td>
<td>0.25</td>
<td>0.25</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>0.10</td>
<td>0.50</td>
<td>0.50</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>0.50</td>
<td>1.00</td>
<td>1.00</td>
<td>50.00</td>
<td>50.00</td>
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<tr>
<td>1.00</td>
<td>2.00</td>
<td>2.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

* Taxol was removed from the medium for 18 h prior to beginning the invasion assay.

The effects of taxol on collagenase synthesis, turnover, and secretion are shown in Table 3. The data showed that the amount of 3H-type IV collagenase [i.e., M, 72,000] immunoprecipitated from the whole cell extracts of taxol-treated cells was drastically reduced from that found in untreated cells (~30,000 ± 300 (SD) cpm). By comparison, the immunoprecipitates from treated cells contained 600 ± 100 cpm after 6 h and 400 ± 50 cpm after 9 h exposure. The amount of total labeled protein in whole cell extracts was 350,000 ± 5,100 cpm in controls, 330,000 ± 4,000 cpm after 6 h exposure, and 295,000 ± 1,500 cpm after 9 h exposure to taxol.

To test further whether taxol influenced type IV collagenase turnover rates, the cells were first labeled (200 µCi/ml [3H]methionine for 6 h) and then exposed to 1.0 µM taxol for 0, 6, and 9 h. Immunoprecipitation measurements revealed cytoplasmic counts (cpm) of 28,000 ± 220 (at 0 h), 25,000 ± 420 (after 6 h), and 24,000 ± 330 (after 9 h) incubation in the presence of taxol. The counts were somewhat lower in the experiments compared to the controls, but overall the data indicated that there was very little reduction of the type IV collagenase levels over a 9-h interval. As an alternative approach, following pulse labeling for 6 h, we measured collagenase turnover rates in cells exposed to serum-free medium containing 10 mg/ml PC-3 ML CM and 1.0 µM taxol (Table 2). The data showed that the cytoplasmic levels of 3H-collagenase dropped from about 25,000 ± 400 cpm to 15,000 ± 300 cpm during the first 3 h. Conversely, the 3H-collagenase levels in the medium rose to about 12,000 ± 300 cpm as a result of secretion in the first 3 h. The levels of 3H-collagenase in the cytoplasm and medium remained fairly constant at levels comparable to that measured at 3 h after 6- and 9-h intervals. We interpret the data to mean that collagenase secretion probably occurs for the first 3 h of exposure to taxol but was then inhibited by 6 and 9 h as a result of the diffusion of taxol in the cells. We conclude that taxol probably has no direct effects on stability and turnover of cytoplasmic collagenase. Also, the PC-3 ML CM does not appear to influence the stability of collagenase in the taxol-treated cells.

It was important to compare if total protein secretion or type IV collagenase secretion were inhibited by taxol in radiolabeled cells. Table 3 shows that the total labeled protein secreted in response to PC-3 ML CM (10 mg/ml) was about 300 ± 200 cpm after 6 h and 200 ± 95 cpm following 9 h preexposure of the cells to taxol, indicating inhibition of total secretion of protein. Control cells which were not treated with taxol secreted total protein counts of ~360,000 ± 200 cpm. Immunoprecipitation measurements of the amounts of 3H-labeled type IV collagenase secreted revealed that the medium contained 50 ± 10 cpm (after 6 h) and 60 ± 20 cpm (after 9 h) exposure to taxol, indicating an inhibition of protease secretion. By comparison, the medium obtained from control cells contained 40,000 ± 400 cpm. In sum, the data showed that taxol inhibited both the synthesis and secretion of the M, 72,000 type IV collagenase. We suggest that when type IV secretion is blocked, translation is somehow inhibited, perhaps by mechanisms dependent on the cytoplasmic processing and packaging of the protease. Interestingly, total protein secretion was also blocked perhaps as a direct result of the disruption of the microtubule distribution.

Boyden Chamber Invasion Studies. Boyden chemotactic assays have been developed previously for studying the ability of tumor cells to invade the basement membrane material (13, 15, 20). Utilizing this approach, we have quantitatively measured the influence of taxol on the ability of the PC-3 ML cells to penetrate Matrigel (Table 4). The measurable levels of invasion
described previously (13). With untreated cells, the mice exhib-

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Table 5 Effects of taxol on PC-3 ML metastases to the lumbar vertebrae
in SCIDs

<table>
<thead>
<tr>
<th>Taxol (µM)</th>
<th>Treatment time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>0.1</td>
<td>18/20</td>
</tr>
<tr>
<td>0.5</td>
<td>17/20</td>
</tr>
<tr>
<td>1.0</td>
<td>18/20</td>
</tr>
</tbody>
</table>

When the cells were pre-exposed to taxol at 0.1 µM for 3 h and 6 h, a substantially reduced number of the mice (4/20 and 1/20, respectively) exhibited metastases to the vertebrae. Tumors were not present in other tissues (i.e., lungs, liver, kidney, spleen, brain) as determined by gross dissections and histology.

Fig. 4, is an H & E image of a tumor in the lumbar vertebrae of a mouse injected with PC-3 ML cells previously exposed to reduced levels of taxol, 0.1 µM taxol for 3 h. The tumor size and appearance (Fig. 4A) was not unlike that observed in mice injected with untreated cells. Moreover, no obvious microtubule bundling was apparent in the mitotic spindles following immunofluorescence labeling with beta tubulin antibodies (Fig. 4B). Therefore, we believe that taxol was not taken up by these cells in significant amounts.

Preexposure of the cells to 0.5 or 1.0 µM taxol for 3 to 6 h prior to injection i.v. completely blocked metastases. Gross dissections and histology revealed that none of the mice had tumor tissue in the vertebrae or other tissues. We believe, that this may result from poor survival, and/or an inability of the cells to invade tissue and/or to grow tumors.

When 10 mice were injected with excess untreated cells (2 x 10^7 cells/ml in 0.2 ml) numerous lung nodules formed by 20 days (13). In mice injected with taxol treated cells (0.5 µM for 6 h), absolutely no tumors were found after 20 days, 30 days and 60 days, indicating cell number and time were probably not limiting factors.

Effect of Taxol on Tumor Growth in Vivo. Previous experience has shown that bone tumors are usually evident after 5 days following the injection of PC-3 ML cells at 2 x 10^7 cells per ml (13). To determine if taxol prevented tumor growth in vivo, SCIDs were injected i.v. with PC-3 ML cells (2 x 10^7 cells/ml in 0.2 ml) and left 5 days. On day 6, the mice were injected i.v. via the tail vein with taxol (50 mg/m^2/day and 250 mg/m^2/day in 0.2 mls). Ten mice were treated with each taxol dosage tested and five control mice received equivalent amounts of polyoxy-

etylated castor oil, the vehicle in which taxol was formulated. After 15 days of treatment, the mice were sacrificed and examined for tumors by dissection and histology. Gross dissection revealed that tumors grew specifically in the lumbar vertebrae (i.e., filling the bone marrow) in all the control mice. The bone was usually destroyed in several areas and the tumors had metastasized into the periosteal cavity. Gross dissection of the taxol treated mice showed that none of the 20 mice exhibited noticeable tumors in any tissues examined (lungs, liver, colon,
Immunofluorescence labeling with beta-tubulin antibodies revealed abnormal mitotic spindles (=15 µm in length) in tumor cells of the taxol treated mice (Fig. 5). Also, the number of mitotic cells detected per field of view were about 5 fold higher than in the controls. Note that it was not technically possible to resolve individual interphase microtubules (or bundles of microtubules) as discrete structures, but the tubulin rich cytoplasm fluoresced as a result of antibody binding to tubulin. The control sections labeled with 2' ab-FITC alone failed to fluoresce. Interestingly, antibody labeling showed that the cells were positive for the p170 multi-drug resistance glycoprotein (data not shown).

To determine if any tumor growth occurred months after the taxol treatment was discontinued, mice were injected i.v. with 2 × 10⁶ cells/ml (0.2 ml), left 5 days and exposed to taxol from day 5 to 15 (i.e., 50 and 250 mg/m²/day). Treatment was discontinued on day 15 and the mice sacrificed 3 months later. Gross dissection and histology revealed that only 40 to 60% of the mice contained tumors; 6/10 (50 mg/m²/day taxol) and 4/10 (250 mg/m²/day taxol). In all cases, the tumors observed were small and delimited to the marrow cavity of the lumbar vertebrae. Immunolabeling of cryosections with tubulin antibodies revealed that the spindles were about 1/3 shorter in length (≈5 µm) than normal spindle (≈7 µm) found in control cells. Taxol treatment may, therefore, have long-term inhibitory effects on cell division and tumor growth. The apparent total eradication of the vertebrae tumors, in at least 20 to 40 percent of the mice tested, is noteworthy and may inadvertently arise from cell death. Note that these latter values were corrected (i.e., the raw data reduced 20%) to account for a potential 20% failure by the PC-3 ML cells to initiate tumors (13).

The dosages tested here were somewhat low in comparison to the levels which have been used in clinical trials (i.e., up to 350 mg/m²/day; 7–11). Unfortunately, if these higher dosages were administered in mice, death or extreme hypersensitivity (vomiting, droopiness) usually occurred in the animals.

In conclusion, the data demonstrates that taxol is of significant importance in the treatment of metastatic bone tumors, including tumors exhibiting the multi-drug resistance phenotype (see Table I). More specifically, we believe that taxol has therapeutic utility in inhibiting molecular processes activated during invasion of the basement membrane and, therefore, may be used to prevent micrometastases in malignant tissue during surgery, radiation or chemotreatment. The future use of taxol
as an anti-cancer drug may be enhanced greatly with the development of non-toxic derivatives, or of receptor or antibody mediated taxol delivery mechanisms to improve tumor uptake and to reduce toxicity. Higher, more effective dosages could then be used without generating severe long-term reactions by patients.

References

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